Structure of the XPC binding domain of hHR23A reveals hydrophobic patches for protein interaction

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Abstract

Rad23 proteins are involved both in the ubiquitin-proteasome pathway and in nucleotide excision repair (NER), but the relationship between these two pathways is not yet understood. The two human homologs of Rad23, hHR23A and B, are functionally redundant in NER and interact with xeroderma pigmentosum complementation group C (XPC) protein. The XPC–hHR23 complex is responsible for the specific recognition of damaged DNA, which is an early step in NER. The interaction of the XPC binding domain (XPCB) of hHR23A/B with XPC protein has been shown to be important for its optimal function in NER. We have determined the solution structure of XPCB of hHR23A. The domain consists of five amphipathic helices and reveals hydrophobic patches on the otherwise highly hydrophilic domain surface. The patches are predicted to be involved in interaction with XPC. The XPCB domain has limited sequence homology with any proteins outside of the Rad23 family except for saccin, a protein involved in spastic ataxia of Charlevoix-Saguenay, which contains a domain with 35% sequence identity.

Keywords: Rad23; xeroderma pigmentosum; NER; DNA repair; NMR structure; chaperone; saccin

Nucleotide excision repair (NER) plays a crucial role in the prevention of mutagenesis and consequent carcinogenesis by the elimination of a wide variety of DNA lesions (Friedberg et al. 1995; Sarasin 1999; Berneburg and Lehmann 2001). NER deficiency in humans is associated with the rare disorder xeroderma pigmentosum (XP). Patients with XP exhibit increased photosensitivity, suffer from skin abnormalities, and have a >1000-fold increased frequency of sunlight-induced skin cancers (Kraemer et al. 1987; Friedberg et al. 1995). About seven different variants of the XP syndrome have been discovered (XP complementation group A to G). The XP group C (XP-C) is particularly interesting because its defect is limited to the global genome repair pathway, which eliminates lesions from the entire genome, and in contrast to other XP groups, it is not involved in the transcription-coupled repair (Friedberg et al. 1995; Sug-

asawa et al. 1998, 2001). All variants of the XP syndrome appear to be caused by defects in proteins that function in NER. The protein factor related to the XP-C-variant of XP has been identified as a complex of the XPC protein and its interaction partner hHR23 (human homolog of the yeast Rad23 protein; Masutani et al. 1994). Thus, understanding how hHR23 interacts with XPC protein is important for elucidating the molecular basis of XP. Recently, some successful trials were performed to reconstitute genetically corrected skin in vitro (Arnaudeau-Begard et al. 2003). A retroviral expression of the wild-type XPC protein in the keratinocytes from patients with XP-C led to restoration of the normal DNA repair and cell survival properties after UV irradiation. This result demonstrates that knowledge about the molecular mechanism of XP syndrome may ultimately be useful in gene therapy.

XPC/hHR23 was shown to bind specifically and preferentially to a number of types of damaged DNA in the early stages of the NER pathway and was consequently identified as an initiator of global genome NER (Sugasawa et al. 1998; Batty et al. 2000; Hey et al. 2002). The XPC component of the XPC/hHR23 complex was shown to be responsible

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for recognition of and binding to damaged DNA, and the hHR23 component has been shown to protect the XPC protein from proteolytic degradation and to stimulate its function (Sugasawa et al. 1996; Ng et al. 2003). Two homologs of hHR23 protein, hHR23A and hHR23B, have been identified. They appear to be functionally redundant in their interaction with XPC and their role in NER (Li et al. 1997; Sugasawa et al. 1997). hHR23A/B consist of four domains: an N-terminal ubiquitin-like domain (UBL), two ubiquitin-associated domains (UBA1 and UBA2), and an XPC binding domain (XPCB) located between UBA domains (Masutani et al. 1997). The UBL and UBA domains link the hHR23 proteins to the ubiquitin/proteasome pathway through specific interactions with the S5a subunit of the proteasome (Schauber et al. 1998) and ubiquitin (Chen et al. 2001; Mueller et al. 2004), respectively. In addition to XPC protein, ubiquitin, and the S5a proteasome subunit, hHR23 proteins have been shown to interact with HIV-1 Vpr protein (Withers-Ward et al. 2000), 3-methyladenine-DNA glycosylase (Miao et al. 2000), p300 (Zhu et al. 2001), and Png1p protein (Suzuki et al. 2001). Thus, hHR23 proteins, which interact with both the NER and ubiquitin-proteasome pathways, seem to be a part of a broader regulatory network. hHR23A has also been shown to down-regulate the p53 protein (Zhu et al. 2001), which in turn is involved in transactivation of XPC protein expression (Adimoolam and Ford 2003). Recently, hHR23A was also shown to interact with MDM2 protein and was suggested to be its partner in the regulation of p53 (Brignone et al. 2004).

The structure of the XPC protein has not yet been determined. The protein consists of 939 amino acid residues and was shown to be insoluble when produced in *Escherichia coli*. Amounts not sufficient for structural studies were produced by using a baculovirus expression system (Reardon et al. 1996). In contrast, both hHR23 homologs (A with 363 and B with 409 amino acid residues) are solubly expressed and folded (Masutani et al. 1997; M. Kamionka and J. Feigon, unpubl.). From the sequence analysis, they were predicted to have modular structure. Thus, we have taken a domain approach to solving the structure of hHR23A. We have previously determined the solution structures of the UBL, UBA1, and UBA2 domains (Dieckmann et al. 1998; Mueller and Feigon 2002, 2003). The recently reported solution structure of the full-length hHR23A protein structure (Walters et al. 2003) confirms that the protein consists of these isolated domains separated by largely unstructured linkers.

In this report, we present the solution structure of the isolated hHR23A XPCB domain. A detailed analysis of the structure provides insight into the structural basis for the interaction between hHR23A and XPC. Using the XPCB domain sequence, we have also performed a search through protein sequence databases. We found that the XPCB domain exhibits 35% amino acid identity to a fragment of

sacsin, a protein involved in spastic ataxia of Charlevoix-Saguenay (SACS). In light of the determined domain structures and their protein interactions, we discuss the possible function of hHR23 proteins in NER.

Results

A conserved sequence of the XPC binding region defines a structural domain

The XPCB of hHR23B was determined by deletion analysis to encompass amino acid residues 275–332, which correspond to residues 231–288 of hHR23A. This fragment was shown to be sufficient to stimulate NER activity of XPC in vitro (Masutani et al. 1997). We initially prepared a construct encompassing all residues located between the two UBA domains (204–319) of hHR23A. The ^{15}N -HSQC spectrum of this construct (204–319) revealed that most of this domain is structured, but peaks corresponding to some residues exhibited intensities indicative of unstructured or flexible regions. We therefore prepared two further protein fragments, encompassing residues 232–286 and 223–317,

Figure 1. ^1H - ^{15}N HSQC spectrum of the ^{15}N -labeled XPCB (223–317) performed at 298K with the assignment. The contours of the side-chain amide protons are shown in blue.

Figure 2. Chemical shift index (CSI)-based secondary structure prediction. Calculations were performed for the (A) α carbon and (B) α hydrogen of each residue. The reference value of the chemical shift for each residue was subtracted from the experimental value and plotted as a function of residue number. Reference values are average values for a particular residue when exhibiting random coil structure. For the α carbon diagram, positive, negative, and close-to-zero values of the chemical shift difference for a residue correspond to α -helix, β -sheet, or random structure, respectively. For the α hydrogen diagram, the opposite is true (Wishart and Sykes 1994).

respectively. The shortest construct (232–286), which corresponds almost exactly to the minimal domain determined for NER activity, appeared to be too short. The ^{15}N -HSQC spectrum (data not shown) revealed that the protein is indeed folded but the peak line widths indicated that it has a tendency to aggregate. We therefore assigned and determined the structure of the middle-size construct 223–317. A ^{15}N -HSQC spectrum of the XPCB (223–317) is shown in Figure 1. The analysis of ^{15}N and ^{13}C NOESY (nuclear Overhauser effect spectroscopy) experiments showed that only residues 230–289 exhibit nuclear Overhauser effect (NOE) contacts, thus contributing to the defined three-dimensional structure. A heteronuclear NOE experiment (data not shown) confirmed that only the region of amino acids conserved between both hHR23 homologs (231–287 for hHR23A) is rigid. More detailed studies of the full-length hHR23A dynamics published recently (Walters et al. 2003) confirm our results.

XPCB adopts a compact helical structure

Secondary structure prediction based on the chemical shift index (CSI) indicated that XPCB is highly helical and sug-

gested the existence of five helices, and residues outside of the 230–289 region were predicted to have a random coil structure (Fig. 2). A high-resolution structure was determined on the basis of 917 NOE-derived distance restraints with a root mean square deviation (rmsd) of 0.46 Å for the backbone and 1.11 Å for the heavy atoms (residues 231–285) (Table 1; Fig. 3). The XPCB structure consists of five amphipathic α -helices encompassing residues 233–237, 240–250, 257–265, 269–273, and 277–284, respectively (Fig. 4). The helices create a hydrophobic interior with hydrophilic residues exposed to the solvent. The only hydrophobic part of the structure that is exposed to the solvent is a loop composed of residues 252–256 (PALLP loop; Figs. 3, 5). The short helix 1 ends with D237 and is followed by glutamine and proline, which introduce a turn so that helix 2 is at an angle of $\sim 30^\circ$ to helix 1. Helices 2 and 3 are separated by a long turn comprising six residues, most of which are hydrophobic and, surprisingly, are exposed to the solvent. The turn results in a change of direction of the backbone chain of $\sim 180^\circ$ and orients helix 3 against the first two helices. Helices 4 and 5 are positioned above the plane formed by the first three helices, resulting in a globular domain structure. Helices 4 and 5 are at an angle of about 40° to each other, with helix 5 oriented almost parallel with helix 3. In the nine lowest energy structures, helices 4 and 5 are separated by a kink or a short 3–10 helix (residues 52–54) that induces a kink; however, in 7 of the 25 structures, helices 4 and 5 appear as one continuous helix. Careful analysis of the regions of the NOESY spectra that give rise to structural restraints for this region did not resolve this structural ambiguity. Incorporation of residual dipolar cou-

Table 1. Restraint and structural statistics for XPC binding domain 230–289

NOE distance restraints (unambiguous and nonredundant only)	
Total	917
Intraresidue	514
Sequential ($ i - j = 1$)	141
Medium range ($ i - j \leq 4$)	167
Long range ($ i - j \geq 5$)	95
Average NOE/residue	15.3
Hydrogen bonds	42
Experimental dihedral angles	29
Additional dihedral angles ^a	53
RDCs ^{a,b}	21
RMSD from average structure for 25 best structures, Å	
Backbone atoms (residues 231–285)	0.46
Heavy atoms (residues 231–285)	1.11
Ramachandran plot, %	
Residues in most favorable region	86.7
Residues in additionally allowed region	11.0
Residues in generously allowed region	1.4
Residues in disallowed region	0.9

^a For details see Materials and Methods.

^b RDCs were used only to check correctness of the protein fold but were not included in the final refinement stage.

Figure 3. Stereo view of the backbone atoms of the ensemble of the best 25 structures with the lowest overall and NOE energies. Side chains of the hydrophobic PALLP loop and F234 are shown in red.

plings (Table 1) in the structure calculations also did not remove the diversity.

Our structure confirms the previous secondary structure predictions based on sequence analysis. The XPCB domain was predicted to consist of a few amphipathic helices (Masutani et al. 1997). The presence of a kink between helices 4 and 5 is supported by the CSI-based predictions as well as secondary structure prediction programs. In the CSI, histidine (H276), which is usually a helix-enhancing residue, shows a negative value for $C\alpha$ and a positive value for $H\alpha$, indicating either a break between helices 4 and 5 or an unusual conformation for this residue (Fig. 2). The protein structure prediction program JPRED (Cuff et al. 1998) predicts the existence of a kink between helices 4 and 5 exactly at position H276. The programs PSIPRED (McGuffin et al. 2000) and PredictProtein: PROFsec (Rost and Liu 2003) both suggest one long helix, comprising our helices 4 and 5, but the parameters describing the quality of the secondary structure prediction (confidence of prediction for PSIPRED and reliability index for PROFsec) have much lower values for H276 than for the neighboring residues, indicating that the kink between the helices cannot be excluded.

Discussion

Comparison to the XPCB structure in the full-length hHR23A

Our high-resolution structure of the isolated XPCB domain of hHR23A shows that it forms a compact globular structure composed of five amphipathic helices with the rmsd values of the backbone and heavy atoms to the mean structure of 0.46 Å and 1.11 Å, respectively (for the best 25 structures). The domain was also recently solved in the context of the full-length hHR23A protein, but its structure was not analyzed in detail (Walters et al. 2003). The accuracy of the two

structures cannot be compared, because the number of restraints used for the structure calculations for hHR23A is given for the full-length construct only, which includes both structured domains and unstructured linker regions. From the deposited coordinates of 12 structures (PDB: 1oqy), we calculated the comparable rmsd values of the backbone and heavy atoms to the mean structure as 0.48 Å and 0.97 Å, respectively (for the same region 231–285). The rmsd between our mean structure and the mean structure determined by Walters and coworkers (2003) is 3.26 Å in the same region. Thus, we conclude that the differences between our structures go beyond the uncertainty of the calculations. The overall fold of both structures is very similar. Two regions in which our structure differs from the structure determined in the full-length hHR23A deserve special attention. First, the structure of XPCB in the full-length protein has a shorter helix 2 and a longer helix 3 than our isolated domain. The differences are in the region between helices 2 and 3, so that the number of residues exhibiting secondary structure in this region stays constant. Helix 3 in the Walters and coworkers (2003) structure includes P256 as the fourth amino acid in the helix. This proline does not necessarily destabilize the helix, because at this position a residue does not require an amide proton to create a potential hydrogen bond (Prajapati et al. 2003). We have recalculated the XPCB structure using the dihedral angles and hydrogen bonds taken from the structure of Walters and coworkers (2003). These calculations resulted in structures exhibiting a high convergence (low rmsd) but with much larger violations of the experimental data (higher overall and NOE energies) than in our own results. Thus, we conclude that our structures better fulfill all restraints derived from our data and therefore are probably more accurate. Second, the authors describe XPCB as consisting of only four helices, where their last helix (helices 4 and 5 in our structure) is strongly bent to form a banana shape. Although we cannot exclude the possibility that a single helix forms in this region, our data are more consistent with the presence of a kink between helices 4 and 5. Finally, Walters and coworkers (2003) describe the surface of the XPCB domain as almost entirely hydrophobic, thus predicting its binding to XPC protein as having a hydrophobic nature. Our analysis of both our and their XPCB structure indicates that the protein surface is highly hydrophilic, because 50% of the residues in the region from 230–289 are hydrophilic and all of them are exposed to the solvent.

Possible binding site for the XPC protein

Binding of hHR23 to XPC was suggested to have hydrophobic character based on the observation that the XPC/hHR23 complex is resistant to high levels of salt (Masutani et al. 1994). Analysis of the XPCB structure reveals that it is built of amphipathic helices (Fig. 4). There is only one

Figure 4. (A) Schematic view of the XPCB domain. The α -helices are shown as cylinders. The boundaries of these secondary structure elements are indicated. (B) Stereo view of ribbon representation of the XPCB structure, illustrating amphipathicity of helices. Hydrophilic and hydrophobic residues are labeled in orange and in white, respectively.

region in the sequence where there are several consecutive hydrophobic residues. The region corresponds to the hydrophobic loop P₂₅₂ALLP₂₅₆ (Fig. 3), between helices 2 and 3, whose side chains are completely exposed to the solvent and which is a part of a larger hydrophobic patch (Fig. 5). We speculate that this region is involved in the interaction with XPC. A surface-exposed phenylalanine (F234) which is highly conserved throughout species (Fig. 6), is located on the part of the hydrophobic surface between helices 1–2–3, which is likely to be the interaction site with XPC. Because of its size, XPC likely interacts by hugging XPCB around the PALLP loop (Fig. 5). We note that in the XPCB domain structure determined in the context of the full-length protein by Walters and coworkers (2003), the hydrophobic patches are smaller and F234 is not a part of any one of them.

One cannot fully exclude the possibility that the XPC–XPCB interaction has a hydrophilic character, however. XPC exhibits a high level of affinity for single-stranded DNA (Sugasawa et al. 1996). It was also recently shown that damaged DNA is able to stabilize the XPC protein (Ng et al. 2003). Because the highly hydrophilic XPCB surface resembles the charged DNA helix, it is possible that ionic interactions might be involved in the XPC–XPCB interaction.

We have attempted to perform some interaction studies on the XPCB and XPC complex. The XPC fragment responsible for hHR23 interaction comprises ~248 residues (494–741; Uchida et al. 2002). This XPC fragment is almost 100% insoluble when produced in *E. coli* (Reardon et al. 1996; M. Kamionka and J. Feigon, unpubl.). Recent studies suggest that XPC is an intrinsically unstable protein and that it requires hHR23 proteins for its stabilization (Ng et al. 2003). We therefore attempted both to refold insoluble XPC in the presence of XPCB and to isolate soluble XPC protein by coexpression with XPCB; however, these experiments were not successful. We also cloned and expressed in *E. coli* a shorter GST-fused fragment of XPC, encompassing 126 residues (529–654), but this fragment was also insoluble. There are some indications that XPC protein is highly toxic when expressed at high levels (Siede and Eckardt-Schupp 1986; Wei and Friedberg 1998). This might be the reason

that XPC expressed in *E. coli* is found exclusively in inclusion bodies.

Possible function of hHR23 proteins

Because hHR23 proteins contain, in addition to an XPCB, both a domain that interacts with the proteasome (UBL) and two domains that interact with ubiquitin (UBA), there has been a lot of confusion about their function in protein degradation and the relationship to the DNA repair pathway. It has been proposed that the hHR23 proteins can work both as a shuttle to the proteasome, thus enhancing protein degradation, and as an inhibitor of ubiquitination and consequently protein degradation (Lambertson et al. 2003). The role of the hHR23 proteins in NER was confirmed as a factor in stabilizing XPC and thus enhancing its function (Ng et al. 2003). The redundancy of two hHR23 homologs (A and B) suggests that the protein has an important role in the cell. Although they appear to be interchangeable in NER (Sugasawa et al. 1997), both hHR23 homologs exhibit small functional differences. XPC isolated from cells is found to be complexed mainly with hHR23B (van der Spek et al. 1996) and only a small amount copurifies with hHR23A (Araki et al. 2001). The fraction of hHR23 bound to XPC is small in comparison to the total amount of hHR23 in the cell, which suggests that the protein has some functions other than those related to NER (Sugasawa et al. 1996). The importance of Rad23 proteins was recently confirmed by knockout studies on mouse mHR23 proteins. Inactivation of both mHR23A and mHR23B caused embryonic lethality. Surprisingly, double knockout cells (*mHR23A*^{−/−} *B*^{−/−}) exhibit a strikingly similar phenotype to *XPC*^{−/−}, suggesting that the major role of Rad23 proteins is in NER (Ng et al. 2003).

A search of protein sequence databases revealed that XPCB domains have sequence similarity almost exclusively to the domains of analogous proteins from other species (Fig. 6B). A notable exception is a 35% sequence identity to a fragment of saccin protein (Fig. 6A; MAXHOM-alignment; Sander and Schneider 1991). Defects in the saccin

protein have been identified as the direct causes of autosomal recessive SACS, a disease that is characterized by absent sensory-nerve conduction, reduced motor-nerve velocity, and hypermyelination of retinal nerve fibers (Klockgether et al. 2000). Surprisingly, hHR23 has also been shown to interact with ataxin-3, another protein of unknown function, which, when mutated, causes another form of ataxia called Machado-Joseph disease or spinocerebellar ataxia type 3 (SCA3; Doss-Pepe et al. 2003). This suggests involvement of hHR23 proteins in other important pathways. We speculate that hHR23 proteins are involved in regulation of protein folding and/or stability of several proteins, for example, XPC, ataxin-3, and p53 (Brignone et al. 2004), regulating their lifetime in the cell. Interestingly, saccin contains the N-terminal domain of Hsp 90 and a DnaJ domain, which are both found in proteins involved in chaperone-mediated protein folding.

XPCB has an unusually large number of conserved hydrophilic residues, in particular glutamines (25% in 60-residue domain; Fig. 6), all of which are exposed to solvent. The ~400-residue hHR23 proteins are both exceptionally stable and soluble when expressed in *E. coli*, whereas even short fragments of XPC are mostly insoluble when expressed either in *E. coli* or in the baculovirus system (Reardon et al. 1996). Thus, it seems likely that the function of the highly hydrophilic XPCB surface is to stabilize XPC in solution, which is in agreement with recently published predictions based on *in vivo* studies (Ng et al. 2003), whereas the hydrophobic patches provide the specific binding site.

Interestingly, deletion of the UBL domain of Rad23 leads to UV sensitivity in yeast, which indicates that the UBL is also involved in NER (Watkins et al. 1993; Russell et al. 1999). The UBL domain interacts specifically with the 19S regulatory subunit of proteasome (S5a subunit in human; Schaubert et al. 1998; Mueller and Feigon 2003). The 19S complex is predicted to have a cellular role independent of proteolysis (Russell et al. 1999) and in particular has been shown to exhibit a chaperone-like activity (Braun et al.

1999). The same activity, which is required to unfold a protein before it is able to enter the narrow opening to the proteolytic subunit of proteasome, can apparently also be used to help a protein to fold correctly. The 19S subunit has also been shown to affect NER independently of Rad23 protein (Gillette et al. 2001). In the light of these facts, we hypothesize that the role of the hHR23 proteins in NER is to bind XPC via its XPCB domain and recruit the S5a regulatory subunit of the proteasome through its UBL domain. The chaperone-like activity of S5a may then function in folding the otherwise intrinsically unfolded XPC protein on the XPCB domain.

Materials and methods

Preparation of proteins

DNA fragments encoding XPCB domain of hHR23A protein were amplified by PCR from the human cDNA library (QUICK-Clone cDNA, Clontech) and cloned as GST fusion proteins into the pGEX2T expression vector (Pharmacia) using BamHI and EcoRI restriction enzymes (Invitrogen). The correctness of the cloning was confirmed by DNA sequencing. Three constructs encompassing the following amino acid residues were prepared: (1) 232–286, (2) 223–317, and (3) 204–319. For protein expression, plasmids were always freshly transformed into the *E. coli* strain BL21gold (Stratagene). Transformed cells were grown in LB medium at 37°C and induced at $OD_{600nm} = 0.6$ –0.7 with 1 mM IPTG. Expressed protein was purified on Glutathione Sepharose (Pharmacia), GST was cleaved off with thrombin (Novagen), and the recombinant protein was finally purified on a Superdex S75 gel filtration column (Pharmacia). Uniformly ^{15}N -labeled and ^{13}C , ^{15}N -labeled proteins were obtained by growing the bacteria in M9 minimal medium containing 1g/L of $^{15}NH_4Cl$ or 1g/L of $^{15}NH_4Cl$ and 2g/L of ^{13}C -glucose (Isotec), respectively. NMR samples contained typically 1 mM protein with 20 mM sodium phosphate (pH 7.3), 122 mM sodium chloride, 0.05% sodium azide, and 5% D_2O . NMR samples for RDC measurements were prepared as described by Ruckert and Otting (2000) using 5% pentaethylene glycol monododecyl ether (C12E5) and n-hexanol mixture.

Figure 5. (A) Views of the van der Waals surface of XPCB. Hydrophilic and hydrophobic residues are in orange and in white, respectively. The residues of PALLP-loop and F234 are labeled. (B) Transparent surface with a ribbon representation of α -helices shown inside.

NMR spectroscopy and structure calculations

The program XPLOR3.1 (Brunger 1992) was used for structure calculations. For each calculation, the simulated annealing procedure started from random coordinates using NOE restraints and experimental dihedral angles shown in Table 1 (RDCs were not included at this stage). For the final calculations, only the region exhibiting NOE signals were taken into account, that is, amino acid residues 230–289. The 50 best energy structures from the set of 100 calculated structures exhibited similar both overall and NOE energies and they were subjected to further energy minimization. Additional dihedral angle restraints (Table 1) were applied in the refinement stage. They were consistent with the secondary structure in the structures calculated in the simulated annealing stage, the NOE data, and the CSI-based secondary structure prediction. These restraints were used exclusively for backbone angles ($\Psi = -47 \pm 20^\circ$, $\Phi = -57 \pm 20^\circ$) and were loose enough not to influence the structure but to improve the convergence only. Table 1 shows statistic data for the 25 best structures. The quality of the structures was finally assessed with the program PROCHECK (Table 1; Laskowski et al. 1993). Further energy

Acknowledgments

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